Expression Analysis of Genes Involved in Collagen Cross-Linking and Its Regulation in Traumatic Anterior Shoulder Instability

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ABSTRACT: The molecular alterations involved in the capsule deformation presented in shoulder instability patients are poorly understood. Increased TGFβ1 acts as a signal for production of matrix macromolecules by fibrogenic cells at joint injury sites. TGFβ1, through its receptor TGFβR1, regulates genes involved in collagen cross-linking, such as LOX, PLOD1, and PLOD2. We evaluated *TGFβ1, TGFβR1, LOX,PLOD1*, and *PLOD2* gene expression in the antero-inferior (macroscopically injured region), antero-superior and posterior regions of the glenohumeral capsule of 29 shoulder instability patients and eight controls. We observed that *PLOD2* expression was increased in the anterior-inferior capsule region of the patients compared to controls. *LOX* expression tended to be increased in the posterior portion of patients. Patients with recurrent shoulder dislocation presented upregulation of *TGFβR1* in the antero-inferior capsule portion and of *PLOD2* in the posterior region. Conversely, *LOX* was increased in the posterior portion of the capsule of patients with a single shoulder dislocation episode. In the antero-inferior, *LOX* expression was inversely correlated and *TGFβR1* was directly correlated with the duration of symptoms. In the posterior region, *PLOD2*, *TGFβ1*, and *TGFβR1* were directly correlated with the duration of symptoms. In conclusion, *PLOD2* expression was increased in the macroscopically injured region of the capsule of patients. Upregulation of *TGFβ1*, *TGFβR1*, and *PLOD2* seems to be related with the maintenance of disease symptoms, especially in the posterior region. *LOX* upregulation seems to occur only in the initial phase of the affection. Therefore, *TGFβ1*, *TGFβR1, LOX*, and *PLOD2* may play a role in shoulder instability. © 2015 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 34:510–517, 2016.

Keywords: shoulder instability; gene expression; TGFβ1 signaling; collagen cross-linking

Shoulder dislocation is a common reason for emergency room visits and accounts for about 45% of all dislocations.[1] Traumatic shoulder dislocations are far more common than intentional and/or non-traumatic forms, which are managed by rehabilitation therapy and are not considered herein. Anterior shoulder dislocations contribute 96–98% of all shoulder dislocations.[1] The incidence of first-time anterior shoulder dislocation ranges from 8 to 8.2/100,000 population/year and the prevalence is about 2%.[1] Shoulder instability (SI) is often observed after the initial episode of shoulder dislocation, with a recurrence rate of up to 100% in young athletes.[2,3]

Wound healing is a complex process that requires deposition and accumulation of newly synthesized structural proteins as well as degradation of old or damaged structures composed mainly of the extracellular matrix (ECM).[4] In a previous study, we observed deregulated expression of collagen genes across the capsule of patients with traumatic anterior shoulder instability.[5] The expression of collagen genes were increased in the antero-inferior (AI), antero-superior (AS), and posterior (P) portions of the capsule in patients compared to controls. These molecular alterations may have a role in collagen fibril structure and in the tissue healing process.

An inflammatory response commonly occurs in the earliest phase of wound healing, followed by new connective tissue matrix deposition.[6] Increases of transforming growth factor β1 (TGFβ1) accompany the acute inflammatory phase and appear to act as a signal modulating the production of matrix macromolecules by fibrogenic cells at the injury site.[7] The TGFβ is activated by proteolytic cleavage.[8] This activity is mediated by 2 signaling receptors, TGFβ receptor I (TGFβ1) and TGFβ receptor 2 (TGFβR2), which dimerize and transduce their signal via their serine threonine kinase activity.[9] TGFBR1 is the central propagator of TGFβ signaling.[10]

In the shoulder capsule of patients with adhesive capsulitis, TGFβ was associated with fibrosis and accumulation of a dense matrix of type I and type III collagen within the capsule.[11,12] Moreover, several studies reported similar molecular alterations in adhesive capsulitis and Dupuytren disease. In Dupuytren disease, the increased expression of TGFβ1 and its receptor TGFβR1 has previously been described.[13,14]

TGFβ1 regulates important collagen-modifying enzymes, such as the lysyl oxidase (LOX)[15] and lysyl hydroxylases 1 and 2 (encoded by *PLOD1* e *PLOD2* genes, respectively).[16–18] LOX plays a key role in the maturation of the ECM. LOX is a secreted, copper-dependent amine oxidase which plays a substantial role in the biogenesis of the connective tissue matrix by oxidizing lysine residues in elastin and collagen, thereby initiating the formation of covalent cross-links

Paulo Santoro Belangero and Mariana Ferreira Leal contributed equally to this study.
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which stabilize these fibrous proteins.[19,20] LOX activity is essential to maintain the tensile and elastic features of connective tissues.[20,21] In many pathological fibrotic situations, the expression of the cross-linked enzyme LOX and its enzymatic activity are controlled by TGFβ1.[15] Additionally, differential variations of TGFβ1 were able to induce the LOX activity in an in vitro model of mechanical injury in ligament cells.[22]

The lysyl hydroxylases promote ECM structural stability and maturation by promoting inter- and intramolecular cross-links and the addition of carbohydrate moieties to ECM molecules.[23,24] Patients with Ehlers–Danlos Syndrome (EDS) type VI present mutations in the *PLOD1* gene, which result in reduced activity of this lysyl hydroxylase. EDS type six is a heritable disorder characterized by kyphoscoliosis, joint laxity, skin fragility, and muscle hypertonia.[25] Therefore, PLOD1 has an important role in joint tissue structure and function. PLOD2 is implicated in several pathological processes, including fibrosis. The increased expression of *PLOD2* was described in fibroblasts isolated from keloid, hypertrophic scars, and the *palmar fascia* of patients with Dupuytren disease.[26] Furthermore, some patients with EDS also present reduced *PLOD2* expression in skin fibroblasts.[27] To our knowledge, no lysyl oxidase, or hydroxylase has been implicated in normal or pathological processes in the glenohumeral capsule.

We hypothesized that gene expression alterations may arise as a result of shoulder dislocation and might be a contributor to recurrent dislocation episodes. We therefore evaluated, for the first time in literature, the expression of *TGFβ1, TGFβR1, LOX*, *PLOD1*, and *PLOD2* mRNA in three regions of the glenohumeral capsule in patients with traumatic anterior shoulder instability and controls. Moreover, we investigated the possible associations between gene expression in capsule samples and preoperative clinical parameters.

METHODS

This is a case-control study (level of evidence three) in which samples of patients with and without shoulder instability were evaluated.

Patients

We evaluated 29 outpatients with traumatic anterior shoulder dislocation from São Paulo Hospital of the Federal University of São Paulo (UNIFESP), Brazil. All patients were treated for at least 2 weeks with shoulder immobilization after the first episode of shoulder dislocation and underwent arthroscopic surgical treatment for shoulder instability. The following inclusion criteria were employed: positive apprehension test, a Bankart lesion on magnetic resonance imaging and no history of previous surgery for an injured shoulder. Patients with clinical signs of posterior and/or multidirectional instability or presenting generalized joint hyperlaxity or hypermobility by Beighton score[28] were excluded. Moreover, patients with associated lesions, such as superior lesion anterior posterior (SLAP) lesions detected during the surgery, were excluded.

In addition, eight subjects who underwent arthroscopically assisted treatment for acromioclavicular dislocation were included as a control group. These patients did not present any history of shoulder instability or positive signs for this injury under anesthesia. Moreover, we did not find any radiological indications of glenohumeral capsule alterations. A standard complete joint examination with scope and probe during arthroscopy confirmed that the controls did not present any other concomitant pathology in the shoulder. All controls were physically active.

Informed consent and the approval of the ethics committee of the UNIFESP were obtained (approval number: CEP 51436) from all patients before data and sample collection. A preoperative questionnaire was given to all patients that included questions regarding demographics, age of onset, number of luxation episodes, duration of symptoms, physical activity, type of work, and other clinical variables. In our sample, physical activity involving the upper limbs included basketball, handball, tennis, swimming, climbing, and the some martial arts (judo, jiu–jitsu and capoeira, a Brazilian martial art).

Tissue Samples

During the arthroscopic procedure, tissue samples were obtained from three sites of the glenohumeral capsule of each patient: AI, AS, and P sites. To minimize the variation of sampling, the tissue specimens were taken by two of the authors (PSB and BE).

The samples were collected as previously described by our group.[29] The biopsy samples of AI and AS sites were obtained with the scope in the posterior portal and the basket grasper in the anterior portal. The AI specimen was taken from the most inferior region of the glenohumeral capsule next to the inferior glenohumeral ligament. The AS specimen was taken in the direction of the anterior portal, below the biceps tendon, in the rotator interval area. The P specimen was obtained during the evaluation of the posterior capsulolabral complex with the scope in the anterior portal and the basket grasper in the posterior portal. The P sample was taken in the direction of the posterior portal.

All tissue specimens were immediately immersed in RNAlater® solution (Qiagen, Germany) and then stored at −20°C until RNA extraction.

RNA Extraction

Total RNA was extracted with an RNeasy® mini kit (Qiagen, Germany) following the manufacturer's instructions. The mechanical lysis step was performed using the Tissue Lyser LT equipment (Qiagen, Germany). RNA concentration and quality were determined using a NanoDrop ND-1,000 spectrophotometer (Thermo Scientifc, Wilmington, DE), and RNA integrity was verified by 1% agarose gel electrophoresis. Aliquots of the total RNA were stored at −80°C until further use.

mRNA Expression Analysis

Gene expression was evaluated by reverse-transcription quantitative polymerase chain reaction (RT-qPCR), which is currently considered to be the gold standard technique for the analysis of mRNA level.[30] First, cDNA was synthesized using a High-Capacity cDNA Archive kit (Life Technologies, Foster City, CA) according to the manufacturer's protocol.

To detect the range of expression of the studied genes, reactions were performed with 80–100 ng of cDNA input using TaqMan Low-Density Array (TLDA) cards (Life Technologies, Foster City, CA) and ViiA 7 Real-Time PCR System (Life Technologies, Foster City, CA). Only inventoried TaqMan Gene Expression Assays (Life Technologies, Foster City, CA) were chosen for gene expression analysis. The final volume in each TLDA well is approximately 1 μl.

The *HPRT1* and *B2M* genes were used as internal controls to normalize the sample input amount, based on our previous study that identified suitable reference genes for the gene expression studies in glenohumeral capsule.[31] All qRT-PCR reactions were performed in triplicate for all target genes (*LOX*: Hs00942480_m1; *PLOD1*: Hs00609368_m1; *PLOD2*: Hs01118190_m1; *TGFβ1*: Hs00998133_m1; *TGFβR1*: Hs00610320_m1) and reference genes (*HPRT1*: Hs02800695_m1; *B2M*: Hs00984230_m1). For each sample, the target and reference genes were assayed on the same card to exclude technical variations.

The relative threshold method (Crt method) was applied, which is a robust method that sets a threshold for each curve individually, based on the shape of the amplification curve, regardless of the height, or variability of the curve during its early baseline fluorescence. The expression of collagen genes across the samples was calculated using the equation ΔCrt, in which [ΔCrt = target gene Crt—the mean of reference genes Crt]. A lower cycle threshold value (Crt) indicates higher gene expression.

Statistical Analysis

All gene expression data (ΔCrt) are shown as the median with the interquartile range (IQR).

We verified the distribution of all continuous variables using the Shapiro–Wilk normality test to determine the appropriate tests for subsequent statistical comparisons. The expression data were not normally distributed. Therefore, the Mann–Whitney test was performed to compare the gene expression between the studied groups and clinical variables, such as gender, number of injuries (1 dislocation episode versus >1 dislocation episode), practice of physical activity involving the upper limbs and type of job (manual versus non-manual job). A χ^2 test was used to compare the gender distribution between cases and controls. Spearman's correlation was applied to evaluate the possible correlation between gene expression and age at surgery or duration of symptoms. A p-value of < 0.05 was considered statistically significant.

RESULTS

Patient Data and Clinical Outcomes

Table 1 shows the clinical outcomes of the shoulder instability patients. In our experimental group, 68.8% of patients with shoulder instability for less than one year (time between the onset and surgery) presented two or more dislocation episodes, whereas 92.3% of patients with shoulder instability for more than one year presented two or more dislocation episodes. The duration of the symptoms was associated with the number of dislocation episodes ($p = 0.008$, Mann–Whitney test).

Although no histological assessment was performed, a macroscopic evaluation during the arthroscopic procedure revealed that all shoulder instability patients presented a more flexible capsular aspect in the AI site.

Among the controls, 7 (87.5%) were males and 1 (12.5%) was female, and the median age at time of surgery was 31.44 years (IQR = 13.5). No significant difference was observed in the distribution of gender between the groups ($p = 1$). In addition, age at the time of surgery was not significantly different between the patients and controls ($p = 0.571$, Mann–Whitney test).

Differences Between the Cases and Controls

Table 2 shows the median and interquartile range of *TGFβ1, TGFβR1, LOX*, *PLOD1*, and *PLOD2* expression in the AI, AS, and P sites of the glenohumeral capsule in the patients and controls. The shoulder instability patients presented increased *PLOD2* expression in the AI portion of the capsule compared to the controls ($p = 0.020$). Moreover, *LOX* expression tended to be increased in the P portion of the capsule in the studied patients ($p = 0.058$). No other significant differences between the samples of cases and controls were detected ($p > 0.05$).

Gene Expression and Clinical Variables of Shoulder Instability Patients

LOX expression was increased [2.57 (1.39) vs 4.51 (0.40); $p = 0.048$] and *TGFβR1* was reduced [1.45 (0.43) vs 0.99 (0.01); $p = 0.048$] in the AI portion of the capsule of male compared to female patients. However, only two patients were female.

With regard to the number of injuries, *TGFβR1* expression was increased in the AI portion of the capsule in patients with recurrent shoulder dislocation compared to patients with a single episode of shoulder dislocation [1.37 (0.32) vs 1.70 (0.74); $p = 0.010$; Figure 1A]. In the P portion of the capsule, *PLOD2* was also upregulated in patients with recurrent shoulder dislocation [2.53 (0.58) vs 2.78 (0.45); $p = 0.049$; Figure 1B]. Conversely, *LOX* was increased in the P portion of the capsule of patients with a single shoulder dislocation episode [2.02 (0.703) vs 2.68 (1.54); $p = 0.025$; Figure 1C]. Furthermore, patients with only one dislocation episode presented increased expression of *LOX* in the P region of the capsule compared with controls [2.02 (0.703) vs 3.36 (1.44); $p = 0.007$]. The expression of *LOX* did not differ between patients with recurrent shoulder dislocations episodes and controls ($p > 0.05$, for all capsule regions).

In the AI region of the capsule, the expression of *LOX* ($\rho = 0.421$; $p = 0.029$; Figure 2A) and *TGFβR1* ($\rho = -0.479$, $p = 0.012$; Figure 2B) were correlated with the duration of symptoms. In the P region of the capsule, the expression of *PLOD2* ($\rho = -0.465$; $p = 0.025$; Figure 2C), *TGFβ1* ($\rho = -0.425$, $p = 0.043$; Figure 2D) and *TGFβR1* ($\rho = -0.446$, $p = 0.032$; Figure 2E) were correlated with the duration of symptoms.

Patients who engage in physical activity involving the upper limbs presented reduced *PLOD1* expression

Table 1. Distribution of the Clinical Outcomes of Shoulder Instability Patients

Variable	Distribution
Age at surgery, years [median (IQR)	28.5 (9)
Age of onset, years [median (IQR)	25 (9)
Gender [N (%)]	
Male	27 (93.1)
Female	2 (6.9)
Duration of condition, years [median (IQR)]	1 (2.1)
Duration of condition [N (%)]	
≤ 1 year	16 (55.2)
> 1 year	13 (44.8)
Number of injuries [N (%)]	
1 dislocation	6 (20.7)
> 1 dislocation	23 (79.3)
Physical activity involving the upper limbs [N (%)]	
No	12 (41.4)
Yes	17 (58.6)
Manual job [N (%)]	
No	20 (69)
Yes	9 (31)

N: number of patients. IQR: interquartile range.

in the AS portion of the capsule [1.31 (0.18) vs 1.06 (0.72); $p = 0.045$; Figure 3A]. In the P portion of the capsule, *PLOD2* expression was also reduced in patients who engage in physical activity involving the upper limbs compared to those that did not practice this type of physical activity [2.75 (0.74) vs 2.44 (0.38); $p = 0.033$; Figure 3B]. A tendency of reduced *TGFβ1* expression in the P portion of the capsule was also observed in these patients [1.02 (0.60) vs 0.76 (0.48); $p = 0.055$; Figure 3C].

No association between the expression of the studied genes and any other clinical variable was found in shoulder instability patients ($p > 0.05$).

DISCUSSION

The AI portion of the glenohumeral capsule of shoulder instability patients commonly exhibits macroscopic alteration,[32,33] such as the capsular deformation that was detected during surgical treatment in all the studied patients. In the present study, we found that *PLOD2* expression was increased in the AI portion of the capsule of patients compared with controls. In several connective tissues (for example, bone, tendon, ligaments, and cartilage)[24] and in fibrotic skin, the formation of collagen cross-links occur by the hydroxyallysine route.[24,34] In this pathway, a hydroxylysine residue in the collagen telopeptide (the terminal non-triple helical domain) is converted into aldehyde hydroxyallysine. Subsequently, the hydroxyallysine reacts with a hydroxylysine residue in the collagen triple helix to form functional cross-links. In contrast to PLOD1, PLOD2 is a lysyl hydroxylase that hydroxylates the telopeptides.[34] Therefore, the shoulder dislocation episode may lead to *PLOD2* up-regulation in an attempt to heal the capsule through the cross-linking of the new collagen fibrils by the hydroxyallysine route in the injured tissue.

Although we did not observe a significant difference between cases and controls in the P portion of the glenohumeral capsule, patients with more than two episodes of shoulder dislocation and with longer duration of symptoms presented increased expression of *PLOD2* in this capsule region. There is a reciprocal load-sharing relationship in the capsule, whereby tensile load in either the anterior or superior structures is simultaneously accompanied by laxity in the P or inferior portion, respectively.[33] The P region of the capsule of anterior shoulder instability patients also presents upregulation of *COL1A1*, *COL1A2, COL3A1*,[5] and other non-collagen ECM genes (unpublished data). Thus, our results reinforce the idea that the molecular alterations noted in the P region of the capsule may be

Table 2. Expression of Genes Involved in Collagen Cross-Linking and Its Regulation in the Glenohumeral Capsule of Patients With Shoulder Instability and Controls

	AI			AS			P		
Gene	Cases [ΔCrt; Median (IQR); $N = 29$]	Controls [ΔCrt; Median (IQR); $N = 8$]	p-value	Cases [ΔCrt; Median (IQR); $N = 29$]	Controls [ΔCrt; Median (IQR); $N = 8$]	p-value	Cases [ΔCrt; Median (IQR); $N = 29$]	Controls [ΔCrt; Median (IQR); $N = 8$]	p-value
LOX	2.64 (1.70)	3.37 (1.52)	0.285	2.65 (1.33)	3.18 (1.98)	0.104	2.55 (1.59)	3.36 (1.44)	0.058[b]
PLOD1	1.08 (0.61)	1.07 (0.47)	0.618	1.29 (0.40)	1.06 (0.72)	0.223	1.19 (0.52)	1.10 (0.42)	0.891
PLOD2	2.64 (0.85)	3.11 (1.08)	0.020[a]	2.66 (0.76)	2.61 (0.65)	0.968	2.59 (0.60)	2.85 (1.70)	0.089
TGFβ1	0.75 (0.58)	0.58 (0.52)	0.605	0.93 (0.49)	0.77 (0.51)	0.133	0.89 (0.48)	0.82 (1.71)	0.785
TGFβR1	1.42 (0.40)	1.47 (0.73)	0.883	1.51 (0.93)	1.61 (0.19)	0.968	1.68 (0.88)	2.08 (0.76)	0.219

AI: antero-inferior portion of the glenohumeral capsule; AS: antero-superior portion of the glenohumeral capsule; P: posterior portion of the glenohumeral capsule; IQR: interquartile range; N: number of samples. A lower delta cycle threshold value (ΔCrt) indicates higher gene expression.[a]Significant difference between groups by Mann–Whitney test ($p < 0.05$). [b]A tendency to increased expression in shoulder instability patients by Mann–Whitney test.

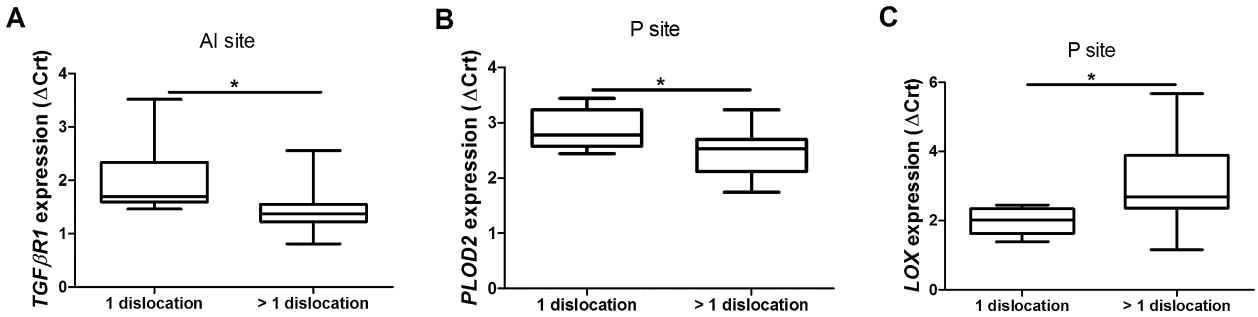

Figure 1. Gene expression by number of dislocation episodes. (A) Increased *TGFβR1* expression in the antero-inferior site of the glenohumeral capsule of shoulder instability patients with more than 1 dislocation episode compared to patients with 1 dislocation episode. (B) Increased *PLOD2* expression in the posterior site of the glenohumeral capsule of shoulder instability patients with more than 1 dislocation episode compared to patients with 1 dislocation episode. (C, B) Increased *LOX* expression in the posterior site of the glenohumeral capsule of shoulder instability patients with 1 dislocation episode compared to patients with more than 1 dislocation episode. 1 dislocation: gene expression in the capsule of patients who have experienced a single episode of shoulder dislocation ($N = 6$). > 1 dislocation: gene expression in the capsule of patients who have experienced more than one episode of shoulder dislocation ($N = 23$). A lower delta cycle threshold value (ΔCrt) indicates higher gene expression. $^{*}p$-value < 0.05 by Mann–Whitney test.

indicative of the biomechanic tissue disorders. Furthermore, the increased *PLOD2* expression in this region may be related to the continuation of the symptoms and their recurrence.

PLOD2, *TGFβ1*, and *TGFβR1* may also have a role in the disease etiology. We observed that the expression of these genes increases with the duration of disease in the P region. *PLOD2* seems to be regulated by *TGFβ1*.[17] Moreover, previous studies have shown that *TGFβR1* is a key element in the regulation of wound healing.[35] Therefore, the continuation of symptoms may contribute to activation of the TGFβ pathway in the P region.

On the other hand, the expression of *LOX* seems to be reduced with the disease. Although we observed that *LOX* expression tended to be increased in the P region of the patient's capsule, its expression was significantly higher in patients with only one dislocation episode compared to controls and compared to patients with recurrent dislocations. Moreover, this gene expression was inversely correlated to the duration of symptoms in the macroscopically injured capsule region. Thus, increased *LOX* expression seems to be occurring in an initial phase of the disease.

Our previous study demonstrated the increased expression of collagen fibrils genes across the capsule

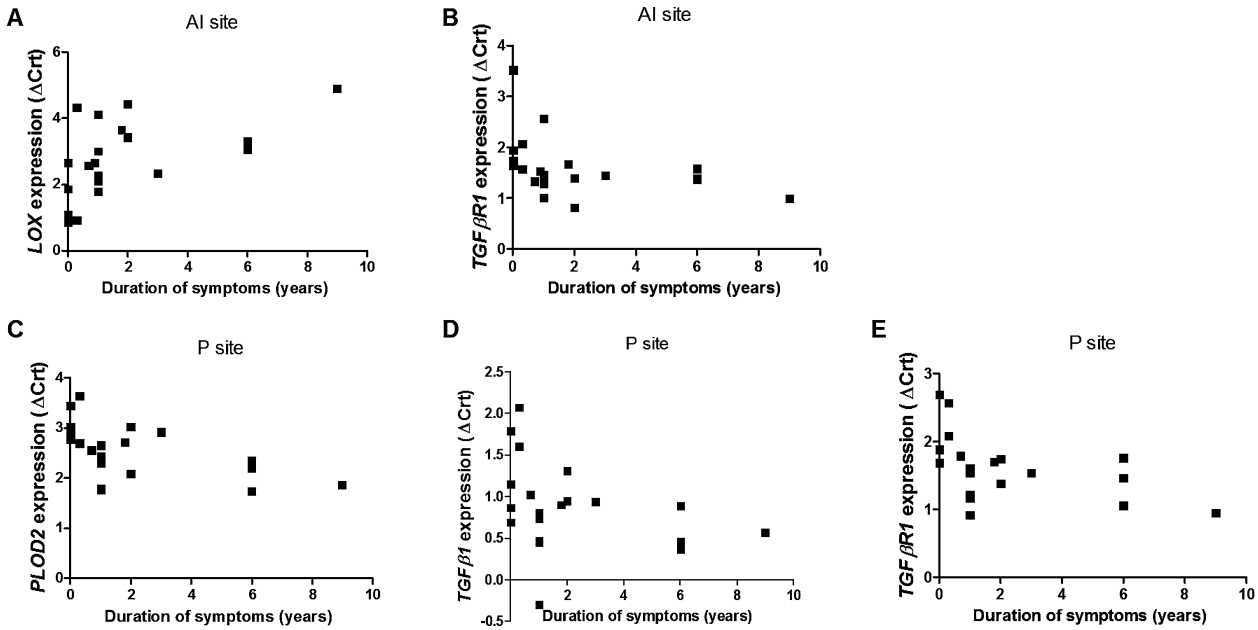

Figure 2. Correlation between gene expression and duration of shoulder instability symptoms (years). (A) *LOX* expression in the antero-inferior site of the glenohumeral capsule. (B) *TGFβR1* expression in the antero-inferior site of the glenohumeral capsule. (C) *PLOD2* expression in the posterior site of the glenohumeral capsule. (D) *TGFβ1* expression in the posterior site of the glenohumeral capsule. (E) *TGFβR1* expression in the posterior site of the glenohumeral capsule. A lower delta cycle threshold value (ΔCrt) indicates higher gene expression; 29 samples of each capsule site was used for each statistical analysis.

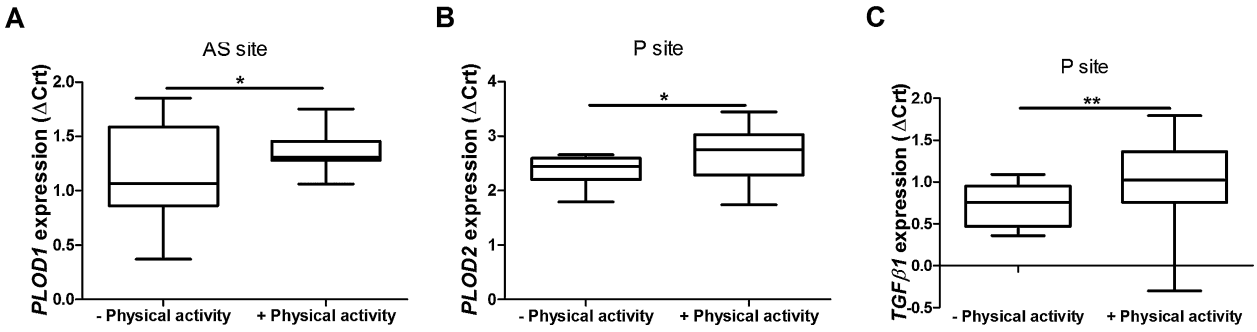

Figure 3. Gene expression by physical activity. (A) Reduced *PLOD1* expression in the antero-superior site of the glenohumeral capsule of shoulder instability patients that practiced physical activity compared to patients that did not practice physical activity. (B) Reduced *PLOD2* expression in the posterior site of the glenohumeral capsule of shoulder instability patients that practiced physical activity compared to patients that did not practice physical activity. (C) A tendency to reduced *TGFβ1* expression in the posterior site of the glenohumeral capsule of shoulder instability patients that practiced physical activity compared to patients that did not practice physical activity. Physical activity: gene expression in the capsule of patients that did not report the practice of physical activity involving the upper limbs ($N = 12$). + Physical activity: gene expression in the capsule of patients that reported the of practice physical activity involving the upper limbs ($N = 17$). A lower delta cycle threshold value (ΔCrt) indicates higher gene expression. $^{*}p$-value < 0.05 by Mann–Whitney. $^{**}p$-value $= 0.055$ by Mann–Whitney.

of shoulder instability patients compared to controls.[5] It is generally accepted that the total amount of enzymatic cross-linking is controlled by the expression of *LOX*. Several previous studies have demonstrated that collagen cross-link formation directly affects the strength of bones, tendons, and ligaments.[36,37] With concomitant collagen upregulation, we should expect *LOX* upregulation across the capsule. Therefore, the lack of *LOX* upregulation suggests that the new collagen fibrils may have reduced resistance to mechanical stress.

We observed that *PLOD2* and TGF*β1* were reduced in the P portion and *PLOD1* was reduced in the AS portion of the capsule of patients who undertook physical activity involving the upper limbs compared to those who did not. It is widely reported that physical activity involving the superior member can lead to biomechanical and structural capsule modifications, such as capsular tightness, especially in the P region.[38] Although additional investigations are still necessary, we hypothesize that these capsule modifications due to physical activity may explain the reduced *PLOD2*, *TGFβ1*, and *PLOD1* expression in the capsule of a subgroup of patients.

To our knowledge, this is the first study to evaluate *TGFβ1*, *TGFβR1, LOX, PLOD1*, and *PLOD2* gene expression in the glenohumeral capsule of shoulder instability patients. However, this study has some limitations. First, we evaluated a single time point (at the time of surgical repair); thus, we were unable to evaluate the dynamic regulation of gene expression. It is not possible to perform a longitudinal gene expression study in a human capsule sample. Therefore, to try to understand the modifications that occur with time, we performed the statistical correlation analyses between gene expression and duration of symptoms. Second, although no significant intra-articular pathology was detected in controls during the arthroscopic examination, it is important to highlight that the plastic deformation of the joint capsule cannot be easily detected through an arthroscopic or clinical exam. One other limitation concerns the tissue biopsy collection. The synovium is adhered to the capsule and cannot be separated from the capsule using arthroscopic instruments. The synovium may also contributes and influences the gene expression findings. Finally, some statistical analyses exhibited reduced power to detect significant differences between groups, and this was most likely due to the high degree of heterogeneity among patients with anterior shoulder instability. Therefore, false-negative results may have occurred.

In conclusion, we found increased *PLOD2* expression in the macroscopically injured region of the glenohumeral capsule of shoulder instability patients. Upregulation of *TGFβ1*, *TGFβR1*, and *PLOD2* seem to be related with the duration of symptoms, especially in the P region of the capsule. *LOX* upregulation seems to occur only in an initial phase of the disease. Therefore, *TGFβ1*, *TGFβR1*, *LOX*, and *PLOD2* may play a role in shoulder instability.

AUTHORS' CONTRIBUTIONS

Author Contributions Statement: PSB, MFL, BE, and MC conceived and designed the experiments. PSB and BE were responsible for sample collection. EAF, CC, CVA, and ACP were involved in clinical data collection. MFL was responsible for the genetic analysis. MFL and MCS were involved in statistical analysis. PSB and MFL were involved in literature search and wrote the first draft of the manuscript. All authors listed have contributed to all subsequent drafts, and have approved the final manuscript.

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